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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/EP96/01464 <b>(22) International Filing Date:</b> 1 April 1996 (01.04.96)  <b>(30) Priority Data:</b> 9508326.7           25 April 1995 (25.04.95)           GB 9513107.4           28 June 1995 (28.06.95)           GB  <b>(71) Applicant (for all designated States except US):</b> SMITHK- LINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GARCON, Nathalie, Marie-Josephe, Claude [FR/BE]; SmithKline Beecham Bio- logicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE). FRIEDE, Martin [GB/BE]; SmithKline Beecham Biologi- cals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE).  <b>(74) Agent:</b> DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, SB House, Great West Road, Brentford, Middlesex TW8 9BD (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> VACCINES CONTAINING A SAPONIN AND A STEROL		
<b>(57) Abstract</b>  The invention relates to a vaccine composition comprising an antigen, an immunologically active saponin fraction and a sterol.		

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## VACCINES CONTAINING A SAPONIN AND A STEROL

The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention relates to  
5 vaccines containing an antigen, an immunologically active fraction derived from the bark of Quillaja Saponaria Molina such as QS21, and a sterol.

Immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree Quillaja Saponaria Molina are known in the art. For  
10 example QS21, also known as QA21, an Hplc purified fraction from the Quillaja Saponaria Molina tree and its method of its production is disclosed (as QA21) in US patent No. 5,057,540. Quillaia saponin has also been disclosed as an adjuvant by Scott et al, Int. Archs. Allergy Appl. Immun., 1985, 77, 409. However, the use of QS21 as an adjuvant is associated with certain disadvantages. For example when QS21 is  
15 injected into a mammal as a free molecule it has been observed that necrosis, that is to say, localised tissue death, occurs at the injection site.

It has now surprisingly been found that necrosis at the injection site can be avoided by use of formulations containing a combination of QS21 and a sterol. Preferred sterols  
20 include  $\beta$ -sitosterol, stigmasterol, ergosterol, ergocalciferol and cholesterol. These sterols are well known in the art, for example cholesterol is disclosed in the Merck Index, 11th Edn., page 341, as a naturally occurring sterol found in animal fat.

In a first aspect the present invention therefore provides a vaccine composition  
25 comprising an antigen, an immunologically active saponin fraction and a sterol. Preferably the compositions of the invention contain the immunologically active saponin fraction in substantially pure form. Preferably the compositions of the invention contain QS21 in substantially pure form, that is to say, the QS21 is at least 90% pure, preferably at least 95% pure and most preferably at least 98% pure.  
30 Other immunologically active saponin fractions useful in compositions of the invention include QA17/QS17. Compositions of the invention comprising QS21 and cholesterol show decreased reactogenicity when compared to compositions in which the cholesterol is absent, while the adjuvant effect is maintained. In addition it is known that QS21 degrades under basic conditions where the pH is about 7 or greater. A  
35 further advantage of the present compositions is that the stability of QS21 to base-mediated hydrolysis is enhanced in formulations containing cholesterol.

Preferred compositions of the invention are those forming a liposome structure. Compositions where the sterol/immunologically active saponin fraction forms an ISCOM structure also form an aspect of the invention.

- 5 The ratio of QS21 : sterol will typically be in the order of 1 : 100 to 1 : 1 weight to weight. Preferably excess sterol is present, the ratio of QS21 : sterol being at least 1 : 2 w/w. Typically for human administration QS21 and sterol will be present in a vaccine in the range of about 1 µg to about 100 µg, preferably about 10 µg to about 50 µg per dose.

10

The liposomes preferably contain a neutral lipid, for example phosphatidylcholine, which is preferably non-crystalline at room temperature, for example egg yolk phosphatidylcholine, dioleoyl phosphatidylcholine or dilauryl phosphatidylcholine. The liposomes may also contain a charged lipid which increases the stability of the liposome-  
15 QS21 structure for liposomes composed of saturated lipids. In these cases the amount of charged lipid is preferably 1-20% w/w, most preferably 5-10%. The ratio of sterol to phospholipid is 1-50% (mol/mol), most preferably 20-25%.

- 20 Preferably the compositions of the invention contain MPL (3-deacylated monophosphoryl lipid A, also known as 3D-MPL). 3D-MPL is known from GB 2 220 211 (Ribi) as a mixture of 3 types of De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem, Montana. A preferred form is disclosed in International Patent Application 92/116556.

- 25 Suitable compositions of the invention are those wherein liposomes are initially prepared without MPL, and MPL is then added, preferably as 100 nm particles. The MPL is therefore not contained within the vesicle membrane (known as MPL out). Compositions where the MPL is contained within the vesicle membrane (known as MPL in) also form an aspect of the invention. The antigen can be contained within the  
30 vesicle membrane or contained outside the vesicle membrane. Preferably soluble antigens are outside and hydrophobic or lipidated antigens are either contained inside or outside the membrane.

- Often the vaccines of the invention will not require any specific carrier and be  
35 formulated in an aqueous or other pharmaceutically acceptable buffer. In some cases it may be advantageous that the vaccines of the present invention will further contain alum or be presented in an oil in water emulsion, or other suitable vehicle, such as for example, liposomes, microspheres or encapsulated antigen particles.

Preferably the vaccine formulations will contain an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen. Antigen or antigenic compositions known in the art can be used in the compositions of the invention, including polysaccharide antigens, antigen or antigenic compositions derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus (especially human) (such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus (for example HSRV F and G proteins or immunogenic fragments thereof disclosed in US Patent 5,149,650 or chimeric polypeptides containing immunogenic fragments from HSRV proteins F and G, eg FG glycoprotein disclosed in US Patent 5,194,595), antigens derived from meningitis strains such as meningitis A, B and C, Streptococcus Pneumonia, human papilloma virus, Influenza virus, Haemophilus Influenza B (Hib), Epstein Barr Virus (EBV), or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and FHA, or derived from parasites such as plasmodium or toxoplasma.

HSV Glycoprotein D (gD) or derivatives thereof is a preferred vaccine antigen. It is located on the viral membrane, and is also found in the cytoplasm of infected cells (Eisenberg R.J. *et al*; J of Viro 1980 35 428-435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately 60 kD. Of all the HSV envelope glycoproteins this is probably the best characterised (Cohen *et al* J. Virology 60 157-166). In vivo it is known to play a central role in viral attachment to cell membranes. Moreover, glycoprotein D has been shown to be able to elicit neutralising antibodies in vivo and protect animals from lethal challenge. A truncated form of the gD molecule is devoid of the C terminal anchor region and can be produced in mammalian cells as a soluble protein which is exported into the cell culture supernatant. Such soluble forms of gD are preferred. The production of truncated forms of gD is described in EP 0 139 417. Preferably the gD is derived from HSV-2. An embodiment of the invention is a truncated HSV-2 glycoprotein D of 308 amino acids which comprises amino acids 1 through 306 naturally occurring glycoprotein with the addition Asparagine and Glutamine at the C terminal end of the truncated protein devoid of its membrane anchor region. This form of the protein includes the signal

peptide which is cleaved to allow for the mature soluble 283 amino acid protein to be secreted from a host cell.

5 In another aspect of the invention, Hepatitis B surface antigen is a preferred vaccine antigen.

As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg antigen (see Tiollais et al, Nature, 317, 489 (1985) and references therein) HBsAg as  
10 herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. In particular the HBsAg may comprise a polypeptide comprising an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L-protein of HBsAg relative  
15 to the open reading frame on a Hepatitis B virus of ad serotype (this polypeptide is referred to as L\*; see EP 0 414 374). HBsAg within the scope of the invention may also include the pre-S1-preS2-S polypeptide described in EP 0 198 474 (Endotronics) or close analogues thereof such as those described in EP 0 304 578 (Mc Cormick and Jones). HBsAg as herein described can also refer to mutants, for example the 'escape  
20 mutant' described in WO 91/14703 or European Patent Application Number 0 511 855A1, especially HBsAg wherein the amino acid substitution at position 145 is to arginine from glycine.

Normally the HBsAg will be in particle form. The particles may comprise for example  
25 S protein alone or may be composite particles, for example (L\*,S) where L\* is as defined above and S denotes the S-protein of HBsAg. The said particle is advantageously in the form in which it is expressed in yeast.

The preparation of hepatitis B surface antigen S-protein is well documented. See for  
30 example, Harford et al (1983) in Develop. Biol. Standard 54, page 125, Gregg et al (1987) in Biotechnology, 5, page 479, EP 0 226 846, EP 0 299 108 and references therein.

The formulations within the scope of the invention may also contain an anti-tumour  
35 antigen and be useful for immunotherapeutically treating cancers.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A.



1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

5

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will  
10 comprise 1-1000 mcg of protein, preferably 2-100 mcg, most preferably 4-40 mcg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

15

The formulations of the present invention maybe used for both prophylatic and therapeutic purposes.

Accordingly in a further aspect, the invention therefore provides use of a vaccine of the  
20 invention for the treatment of human patients. The invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient. In particular, the invention provides a method of treating viral, bacterial, parasitic infections or cancer which comprises administering an effective amount of a vaccine of the present invention to a patient.

25

The following examples and data illustrates the invention.

## Examples

### 1.1 Method of preparation of liposomes:

5

A mixture of lipid (such as phosphatidylcholine either from egg-yolk or synthetic) and cholesterol in organic solvent, is dried down under vacuum (or alternatively under a stream of inert gas). An aqueous solution (such as phosphate buffered saline) is then added, and the vessel agitated until all the lipid is in suspension. This suspension is then microfluidised until the liposome size is reduced to 100 nm, and then sterile filtered through a 0.2 µm filter. Extrusion or sonication could replace this step.

10

Typically the cholesterol:phosphatidylcholine ratio is 1:4 (w/w), and the aqueous solution is added to give a final cholesterol concentration of 5 to 50 mg/ml. If MPL in organic solution is added to the lipid in organic solution the final liposomes contain MPL in the membrane (referred to as MPL in).

15

The liposomes have a defined size of 100 nm and are referred to as SUV (for small unilamellar vesicles). If this solution is repeatedly frozen and thawed the vesicles fuse to form large multilamellar structures (MLV) of size ranging from 500nm to 15 µm. The liposomes by themselves are stable over time and have no fusogenic capacity.

20

### 1.2 Formulation procedure:

25

QS21 in aqueous solution is added to the liposomes. This mixture is then added to the antigen solution which may if desired contain MPL in the form of 100nm particles.

### 1.3 The lytic activity of QS21 is inhibited by liposomes containing cholesterol.

30

When QS21 is added to erythrocytes, they lyse them releasing hemoglobin. This lytic activity can also be measured using liposomes which contain cholesterol in their membrane and an entrapped fluorescent dye, carboxyfluorescein - as the liposomes are lysed the dye is released which can be monitored by fluorescence spectroscopy. If the fluorescent liposomes do not contain cholesterol in their membrane no lysis of the liposomes is observed.

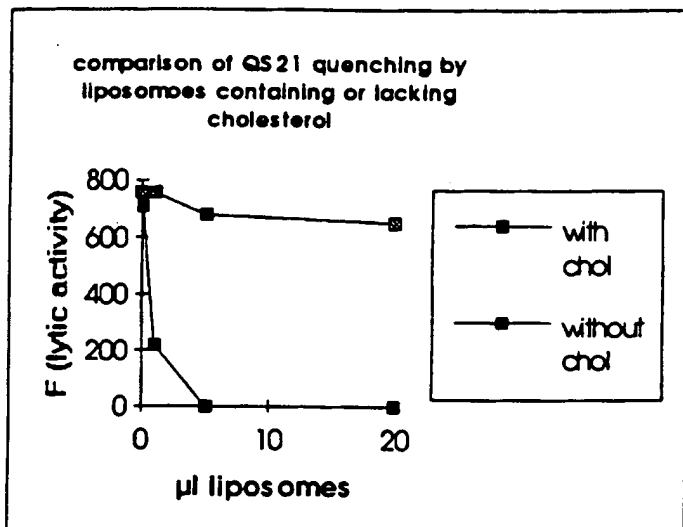
35

If the QS21 is incubated with liposomes containing cholesterol prior to adding it to erythrocytes, the lysis of the erythrocytes is diminished depending on the ratio of cholesterol to QS21. If a 1:1 ratio is used no lytic activity is detected. If the

liposomes do not contain cholesterol, inhibition of lysis requires a one thousand fold excess of phospholipid over QS21.

The same holds true using fluorescent liposomes to measure the lytic activity.

- 5 In the graph below, the lytic activity of 4  $\mu$ g of QS21 treated with liposomes lacking cholesterol (1 mg egg yolk lecithin per ml) or containing cholesterol (1 mg lecithin, 500  $\mu$ g cholesterol per ml) was measured by fluorescence.



10

The data shows that QS21 associates in a specific manner with cholesterol in a membrane, thus causing lysis (of cells or fluorescent liposomes).

If the QS21 first associates with cholesterol in liposomes it is no longer lytic towards cells or other liposomes. This requires a minimum ratio of 0.5:1 chol:QS21(w/w).

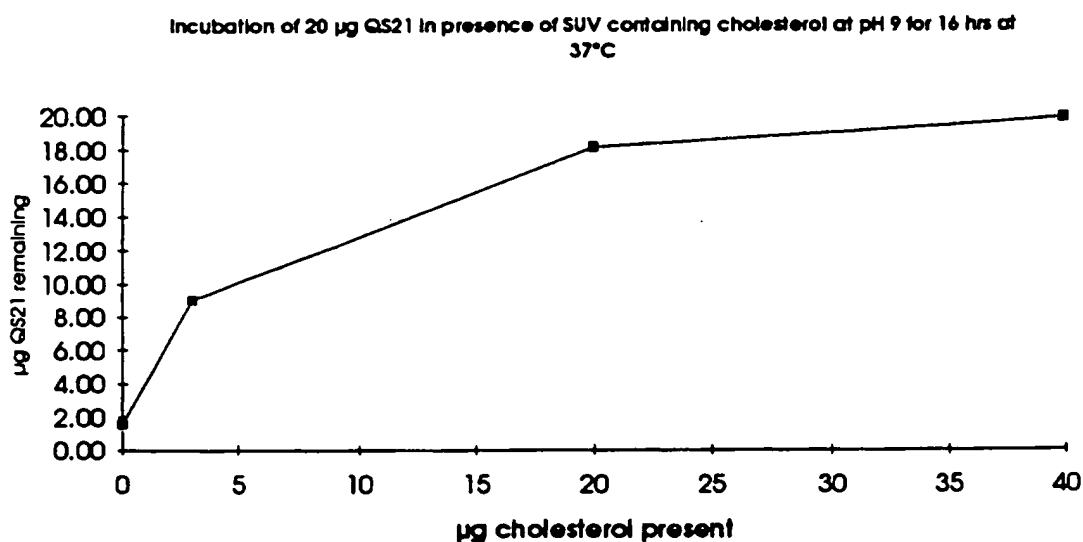
15

Cholesterol is insoluble in aqueous solutions and does not form a stable suspension. In the presence of phospholipids the cholesterol resides within the phospholipid bilayer which can form a stable suspension of vesicles called liposomes. To avoid the requirement to add phospholipids a soluble derivative was tried. Polyoxyethanyl-  
 20 cholesterol sebacate is soluble in water at 60 mg/ml however even at a 2000 fold excess (w/w) over QS21 no reduction in the lytic activity of QS21 was detected.

#### 1.4 Increased stability of QS21 by liposomes containing cholesterol.

5 QS21 is very susceptible to hydrolysis at a pH above 7. This hydrolysis can be monitored by measuring the decrease in the peak corresponding to QS21 on reverse-phase HPLC. For example, the graph below shows that at pH 9 and at a temperature of 37°C, 90% of QS21 is hydrolysed within 16 hours. If liposomes containing cholesterol are added to the QS21 at a ratio of 2:1 (chol:QS21 w/w) no hydrolysis of the QS21 is detected under the same conditions. If the ratio is 1:1 10% of the QS21 is degraded.

10



It is concluded that when QS21 associates with liposomes containing cholesterol it becomes much less susceptible to base-mediated hydrolysis. The hydrolysis product is described as having no adjuvant activity when given parenterally, hence vaccines containing QS21 have to be formulated at acidic pH and kept at 4°C to maintain adjuvant composition. The use of liposomes may overcome this requirement.

#### 20 1.5 Reactogenicity studies:

Mice injected in tibialis muscle with 5 µg QS21 (or digitonin) added to increasing quantities of liposomes (expressed in terms of µg cholesterol). Lytic activity is expressed as µg QS21 equivalent, which means that quantity of QS21 required to achieve the same hemolysis as the sample.

Redness, necrosis and toxicity in the muscle at the site of injection were scored visually after sacrificing the mice.

formulation	lytic activity $\mu$ g	redness	necrosis	toxicity
QS21 +PBS	5	+++	$\pm$	+++
QS21 +1 $\mu$ g chol (SUV)	4	+++	+	++++
QS21 +5 $\mu$ g chol (SUV)	0	-	-	$\pm$
QS21+25 $\mu$ g chol (SUV)	0	$\pm$	-	+
SUV alone	0	-	-	-
digitonin	5	-	-	$\pm$
PBS	0	-	-	-

- 5 The data shows that when the lytic activity is abolished by the addition of liposomes containing cholesterol the toxicity due to the QS21 is also abolished.

#### 1.6 Reactogenicity intra-muscularly in rabbits

10

Values in U.I./L

Experiment	Formulation	Day0	hemolysis	Day1	hemolysis	Day3	hemolysis
Rabbit n°1	QS21 50 $\mu$ g	1078	$\pm$	8650		1523	
Rabbit n°2		1116		4648		1435	
Rabbit n°3		660		4819		684	
Rabbit n°4		592		5662		684	
Rabbit n°5		3400		7528		1736	
Mean		1369		6261		1212	
SD		1160		1757		495	

Experiment	Formulation	Day0		Day1		Day3	
			hemolysis		hemolysis		hemolysis
Rabbit n°6	QS21 50µg Chol in SUV 50µg (1:1)	596	±	1670		460	
Rabbit n°7		540		602		594	
Rabbit n°8		611		1873		803	
Rabbit n°9		521		507		616	
Rabbit n°10		1092		787		555	
Mean		672		1088		606	
SD		238		636		125	

Experiment	Formulation	Day0	hemolysis	Day1	hemolysis	Day3	hemolysis
Rabbit n°11	QS21 50µg Chol in SUV 150µg (1:3)	332		344		387	
Rabbit n°12		831		662		694	
Rabbit n°13		464		356		519	
Rabbit n°14		528		720		614	
Rabbit n°15		1027		568		849	
Mean		637		530		613	
SD		285		173		175	

Experiment	Formulation	Day0	hemolysis	Day1	hemolysis	Day3	hemolysis
Rabbit n°16	QS21 50µg Chol in SUV 250µg (1:5)	540	±	769		745	
Rabbit n°17		498		404		471	
Rabbit n°18		442		717		(4535)	
Rabbit n°19		822		801		925	
Rabbit n°20		3182		2410		960	
Mean		1097		1020		775	(1527)
SD		1175		793		224	(1692)

Experiment	Formulation	Day0	hemolysis	Day1	hemolysis	Day3	hemolysis
Rabbit n°21	PBS	321		290		378	
Rabbit n°22		660		535		755	
Rabbit n°23		650		603		473	
Rabbit n°24		1395		(3545)		(5749)	
Rabbit n°25		429	±	323		263	
<b>Mean</b>		<b>691</b>		<b>438</b>	(1059)	<b>467</b>	(1523)
<b>SD</b>		<b>419</b>		<b>155</b>	(1396)	<b>210</b>	(2369)

The data shows that the addition of cholesterol-containing liposomes to the formulation significantly reduces the elevation in CPK (creatine phospho kinase) caused by the QS21. Since the CPK increase is a measure of muscle damage this indicates decreased muscle damage and is confirmed by the histopathology.

#### 1.7 Binding of the liposome-QS21 complex to alum.

10

QS21 was incubated with neutral liposomes containing excess cholesterol as well as radioactive cholesterol and then incubated with alum ( $\text{Al}(\text{OH})_3$ ) in PBS. Alone, neither neutral liposomes nor QS21 bind to alum in PBS, yet negatively charged liposomes do. When together however, QS21 and neutral liposomes bind to alum. The supernatant contained neither QS21 (assayed by orcinol test) nor radioactive cholesterol.

15

This indicates that the QS21 has bound to the liposomes and permits the liposome-QS21 combination to bind to the alum. This may arise from a negative charge being imposed on the liposomes by the QS21, or to an exposure of hydrophobic regions on the liposomes. The results also imply that QS21 does not extract cholesterol from the membrane.

20

This indicates that compositions of the invention can be used in alum based vaccines.

#### 25 1.8 Comparison of liposomal QS21/MPL and free QS21+MPL for antibody and CMI induction

SUV were prepared by extrusion (EYPC:chol:MPL 20:5:1).

For MPL out, liposomes were prepared without MPL and MPL added as 100 nm. particles

QS21 was added prior to antigen. Chol:QS21 = 5:1 (w/w)

MLV were made by freeze-thawing SUV 3x prior to antigen addition.

- 5 To have the antigen entrapped, the antigen was added to SUV prior to freeze-thawing and QS21 added after freeze-thaw. Antigen encapsulation = 5% in, 95% out.

-mice (balb/c for gD, B10BR for RTSs) were injected twice in the footpad.

gD is the glycoprotein D from Herpes Simplex virus. RTSs is the Hepatitis B surface antigen (HBsAg) genetically modified to contain an epitope from the Plasmodium

- 10 falciparum sporozoite.

ag = 10 µg RTSs	anti HBsAg Titres 15days post boost		
formulation	IgG1	IgG2a	IgG2b
SUV/QS + MPL <sub>(out)</sub> + Ag	1175	10114	71753
MLV/QS + MPL <sub>(out)</sub> + Ag	2247	11170	41755
MLV/QS/MPL <sub>(in)</sub> + Ag	969	7073	18827
MLV/QS/MPL <sub>(in)</sub> /Ag <sub>(in)</sub> + Ag	1812	2853	9393
QS + MPL + Ag	372	9294	44457
Ag	<100	<100	<100
SUV/QS + MPL <sub>(out)</sub>	<100	<100	<100
MLV/QS/MPL <sub>(in)</sub>	<100	<100	<100

ag = 20µg gD	anti-gD	CMI	
formulation	IgG	IFN-γ96 hr (pg/ml)	IL2 48hr pg/ml
SUV/QS + MPL <sub>(out)</sub> + Ag	2347	1572	960
SUV/QS/MPL <sub>(in)</sub> + Ag	2036	1113	15
MLV/QS + MPL <sub>(out)</sub> + Ag	1578	863	15
MLV/QS/MPL <sub>(in)</sub> + Ag	676	373	15
MLV/QS/MPL <sub>(in)</sub> /Ag <sub>(in)</sub> + Ag	1064	715	15
QS + MPL + Ag	1177	764	15
Ag	<100	567	44
SUV/QS + MPL <sub>(out)</sub>	<100	181	15
MLV/QS/MPL <sub>(in)</sub>	<100	814	105



- The data shows that SUV/QS21+MPL<sub>(out)</sub> induces high antibody titres at least as good as QS21+MPL, as well as inducing IL2 a marker of cell mediated immunity, while quenching QS21 reactogenicity.

- Additional results from a second experiment comparing QS21 and QS21 in the presence of cholesterol (SUV) in balb/c mice with HSV gD as antigen are shown below:

Formulation	antigen	Isotypes 7days post II							
		IgG 7 post II (GMT)	IgG 14post II (GMT)	IgG1		IgG2a		IgG2b	
				µg/ml	%	µg/ml	%	µg/ml	%
SUV/QS21 + MPL out	gD (5µg)	20290	16343	331	26	716	56	222	17
SUV/QS21/MPLin	gD (5µg)	12566	10731	418	44	412	44	111	12
QS21+MPL	gD (5µg)	10504	10168	200	34	285	48	107	18
SUV/QS21 + MPL out	none	0	0	0	0	0	0	0	0
QS21	gD (5µg)	3468	4132	156	66	67	28	14	6
SUV/QS21	gD (5µg)	11253	11589	484	57	304	36	65	8

### 1.9 Comparison of gp120 plus liposomal MPL/QS21 with free MPL/QS21

- Liposomes = SUV containing MPL in the membrane  
Chol:QS21 = 6:1

Response was tested two weeks after one immunisation

formulation	proliftn	IFN-g ng/ml	IL2 pg/ml	IL5 pg/ml
SUV/MPL/QS21 + Ag	12606	16.6	59	476
MPL+QS21+Ag	16726	15.8	60	404

After second immunisation:

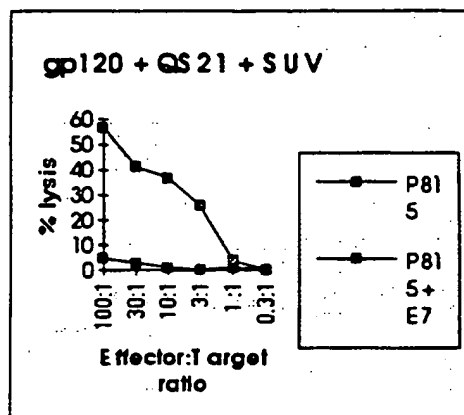
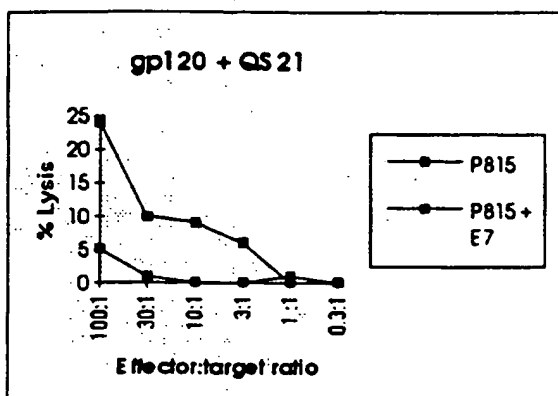
formulation	proliftn	IFN-g ng/ml	IL4 pg/ml	IL5 pg/ml
SUV/MPL/QS21 + Ag	12606	135	0	250
MPL+QS21+Ag	16726	60	0	500

The data shows that QS21 associated with cholesterol-containing liposomes and MPL induces Th1/Th0 response equal to MPL+QS21.

At this ratio of cholesterol to QS21, QS21 is non-toxic in rabbits (by CPK).

5

In a second experiment balb/c mice were immunised intra-footpad with gp120 in the presence of QS21 or QS21 + SUV containing cholesterol. The cytotoxic T-lymphocyte activity in spleen cells was measured.



10

This demonstrates that QS21 alone induces CTL activity, and that QS21 in the presence of liposomes containing cholesterol induces CTL activity at least as good as, or better than, QS21 alone.

15

## 2. Vaccines

### 2.1 Formulation of HBsAg L\*,S particles.

HBsAg L\*,S particles may be formulated as follows:

20

10µg of HBsAg L\*,S particles/dose are incubated 1h. at room temperature under agitation. The volume is adjusted using water for injection and a PBS solution and completed to a final volume of 70µl/ dose with an aqueous solution of QS21 (10µg/dose). pH is kept at  $7 \pm 0.5$ .

25

Similar formulations may be prepared using 1 and 50µg of HBsAg L\*,S and also using the HBsAg S antigen.

These formulations may be tested in the Woodchuck surrogate therapeutic model using Woodchuck HBV antigens as a model.

30

## Woodchuck model

DQ QS21 (i.e. QS21/cholesterol or quenched QS21) may be tested in the woodchuck therapeutic model where animals are chronically infected with the virus. Specific woodchuck hepatitis virus vaccine may be add mixed with QS21 as such or DQ and with or without MPL and administered to the animals every months for 6 months. Effectiveness of the vaccine may be assess through viral DNA clearance.

## 2.2 Guinea Pig Model (HSV)

### 2.2.1 Prophylactic model

Groups of 12 female Hartley guinea pigs were either injected intramuscularly on day 0 and day 28 with the following formulations:

1st experiment:

gD 5 µg + QS21 50 µg + SUV containing 50 µg cholesterol  
 gD 5 µg + QS21 100 µg + SUV containing 100 µg cholesterol  
 gD 5 µg + QS21 50 µg + SUV containing 250 µg cholesterol  
 gD 5 µg + QS21 50 µg

2nd experiment:

gD 5µg + MPL 12.5 µg + QS21 12.5µg + SUV containing 62.5 µg cholesterol, or left untreated.

The animals were bled at 14 and 28 days after the second immunisation, and the sera tested for their gD-specific ELISA antibody titres.

Animals were then challenged intravaginally with 10<sup>5</sup> pfu HSV-2 MS strain. They were scored daily from day 4 to 12 for evaluation of primary herpetic lesions. Scoring was as follows:

Vaginal lesions:

- bleeding = 0.5
- redness for one or 2 days without bleeding = 0.5
- redness and bleeding for a day = 1
- redness without bleeding lasting at least 3 days = 1

External herpetic vesicles:

- < 4 small vesicles = 2
- ≥ 4 small vesicles or one big vesicle 4 ≥ 4 large lesions 8 fusing large lesions = 16
- fusing large lesions on all external genital area = 32.

The results are shown in the table below:

## Prophylactic Model

Experiment 1 (chol refers to SUV containing cholesterol)

FORMULATION		PRIMARY DISEASE						
n		Animal without lesion %	Vaginal lesions incidence %	External lesions incidence %	PI Index **	reduction vs Control	Lesion severity * Median	n
12	gD / QS21 50µg	50	33	17	73	93%	1.50	6
11	gD / QS21 50 µg -chol 1/5	64	18	18	67	93%	2.50	4
12	gD / QS21 50 µg-chol 1/1	100	0	0	0	100%	-	-
12	gD / QS21 50 µg-chol 1/1	50	33	17	54	95%	0.75	6
12	UNTREATED	25	0	75	996	-	55.00	9

Experiment  
2

n	FORMULATION	PRIMARY DISEASE						
		Ab titres (GMT)		Animal without lesion %	Vaginal lesions incidence %	External lesions incidence %	PI Index **	Lesion severity * Median
		ELISA	NEUTRA					
		day 14 post II	day 28 post II					n
12	gD / QS21 / SUV / MPL	47006	31574	58.33	33.33	8.33	37.50	1.00
12	UNTREATED	<400	<400	16.67	8.33	75.00	587.50	11.50
								5
								10

\* Sum of the lesion scores for the days 4 to 12 post-infection (animals without lesion are not considered).

Lesion scores: no lesion (0), vaginal lesions (0.5 or 1), external skin vesicles (2, 4, 8 or 16)

\*\*Primary infection index = SUM (Max.score i) x (Incidence %) ; with i = 0, 0.5, 1, 2, 4, 8 or 16

5 The table and graph show that in the prophylactic model, a very high level of protection against primary disease was induced upon immunisation with gD / MPL / QS21 / SUV. Both the incidence of external lesions and the lesion severity appeared highly reduced in the group of animals immunised with gD / MPL / QS21 / SUV.

### 10 2.2.2 Therapeutic Model

In the therapeutic model, female Hartley guinea pigs were first challenged with 10<sup>5</sup> pfu HSV-2 MS strain. Animals with herpetic lesions were then randomly allotted to groups of 16.

15 On day 21 and day 42, they were either immunised with one of the following formulations:

- gD + MPL 50µg + QS21 50µg + SUV containing 250 µg cholesterol,
- 20 - gD + Al(OH)<sub>3</sub> + MPL 50µg + QS21 50µg, + SUV containing 250 µg cholesterol or left untreated.

They were monitored daily from day 22 to 75 for evaluation of recurrent disease. Scoring was as described for the prophylactic model. The results are shown in the table and graph below:

25

### Therapeutic Model

n	FORMULATIONS	SEVERITY *		DURATION **		EPISODE NBER ***	
		Median	% reduction vs Control	Median	% reduction vs Control	Median	% reduction vs Control
16	gD+MPL+QS21+S	9.00	43%	7.00	18%	3.00	14%
15	gD+AI(OH)3+MPL+QS21+SUV	8.50	46%	7.00	18%	3.00	14%
16	Untreated	15.75	-	8.50	-	3.50	-

\* Sum of the lesion scores for the days 22 to 75 post-infection .

\*\* Total days animals experienced recurrent lesions for the days 22 to 75 post infection

\*\*\* Recurrence episode number for the days 22 to 75 post infection. One episode is preceded and followed by a day without lesion and characterized by at least two days with erythema (score=0.5) or one day with external vesicle (score>=2) Immunotherapeutical treatment performed on days 21 and 42.

The results show that good levels of protection were also induced in the therapeutic model of HSV-2 infection. Immunization with gD / MPL / QS21/SUV with or without Alum had a marked effect on the median severity of recurrent disease. It also slightly reduced episode number and duration (see Table).

## Claims

1. A vaccine composition comprising comprising an antigen, an immunologically active saponin fraction and a sterol.
- 5 2. A vaccine composition according to claim 1 wherein the immunologically active saponin fraction is QS21.
3. A vaccine composition according to claim 1 or 2 wherein the sterol is cholesterol.
- 10 4. A vaccine composition according to claim 2 or 3 wherein the ratio of QS21:sterol is from 1:100 to 1:1.
- 15 5. A vaccine composition according to any of claims 1 to 4 which further contains MPL.
6. A vaccine composition as claimed herein comprising an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline  
20 Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.
- 25 7. A vaccine as claimed in any of claim 1 to 5 wherein the antigen is a tumour antigen.
8. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the prophylatic treatment of viral, bacterial, or parasitic infections.
- 30 9. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the immunotherapeutic treatment of viral, bacterial, parasitic infections or cancer.
- 35 10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.



11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.
- 5 12. A process for making a vaccine composition according to claim 1 comprising admixing an immunologically active saponin fraction and cholesterol with an antigen or antigenic composition.

## INTERNATIONAL SEARCH REPORT

International Application No

PC1/EP 96/01464

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/39 A61K39/02 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 415 794 (COOPERS ANIMAL HEALTH) 6 March 1991	1-4,6-12
Y	see page 2, line 1 - page 4, line 24 ---	5
X	WO,A,92 06710 (NEDERLANDEN STAAT) 30 April 1992	1-4,6-12
Y	see page 1, line 3 - line 9 see page 11, line 13 - page 12, line 2 ---	5
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Y	see page 2, line 19 - page 5, line 23 see page 10, line 6 - line 19 see page 14, line 30 - page 15, line 9 --- -/--	5

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

22 July 1996

Date of mailing of the international search report

31.07.96

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Sitch, W

# INTERNATIONAL SEARCH REPORT

International Application No

PC1/EP 96/01464

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO,A,94 19013 (SMITHKLINE BEECHAM CORP ;SMITHKLINE BEECHAM BIOLOG (BE); DILLON SU) 1 September 1994 see page 3, line 36 - page 4, line 15 see page 16, line 14 - line 24 see page 23, line 31 - page 25, line 14 ---	5
E	WO,A,96 11711 (ISCOTEC AB ;COX JOHN COOPER (AU); COULTER ALAN ROBERT (AU); MOREIN) 25 April 1996 see the whole document -----	1-4,6, 8-12

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Information on patent family members

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PC1/EP 96/01464

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## INTERNATIONAL SEARCH REPORT

national application No.

PCT/EP 96/01464

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 10 and 11 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC/EP 96/01464

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